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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/53, 33/543

A1

(11) International Publication Number: **WO 98/23956**

(43) International Publication Date: 4 June 1998 (04.06.98)

(21) International Application Number: PCT/GB97/03289

(22) International Filing Date: 28 November 1997 (28.11.97)

(30) Priority Data:
9624750.7 28 November 1996 (28.11.96) GB

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(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: CAPTURE ASSAYS

(57) Abstract

A process for testing for the presence of at least two immunological markers in a sample, at least one of which markers is an antigen, which process comprises incubating the sample with a solid support having immobilised thereon capture agents for each of the immunological markers so as to bind any of the immunological markers present in the sample to the solid support, contacting the solid support with labelled binding partners, for each of the immunological markers and detecting the presence of any label or labels bound to the solid support.

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Capture Assays

The present invention relates to one-pot tests able to detect the presence of any of two or more markers in a single sample.

Immunological tests or assays for individual antigens or antibodies are well known. With the ever increasing demands for screening of blood donations to prevent transmission of diseases by blood transfusion there is an increasing need for tests which simultaneously detect multiple markers. Some protocols which are already in use are γ-and μ-and α-specific antibody assays and the recent G-antibody capture assays. These rely on an immobilised anti-antibody as capture agent which, on incubation with a liquid sample containing antibodies, will bind and thus immobilise antibodies of the class recognised by the capture agent. The immobilised antibodies are then probed using labelled test antigen(s) in order to detect the presence of antibodies against the test antigen(s) in the original sample. These systems have a number of disadvantages in the range of markers which may be detected. Thus they only detect antibodies in the sample and the nature of the capture agent restricts the types of antibodies which can be detected.

The present invention provides an alternative test system which permits a wide range of markers to be detected simultaneously in a one-pot reaction. This is based on the use of a mixture of antibodies as capture agent.

Accordingly the present invention provides a

process for testing for the presence of at least two immunological markers at least one of which is an antigen in a sample which process comprises incubating the sample with a solid support having immobilised thereon capture agents for each of the immunological markers so as to bind any of the immunological markers present in the sample to the solid support, optionally washing the solid support, and contacting the solid support with labelled binding partners for each of the immunological markers. This process is suitably followed by further optional washing steps and by the detection of the presence of any label or labels bound to the solid support.

The process of the present invention may be conducted in a single vessel, such as an individual well of a microtitre plate. The solid support may be any conventional solid phase material to which capture agents such as antibodies can be bound, for instance the walls of the vessel or beads contained in the vessel. Capture agents for the immunological markers are obtained and immobilised on the solid support in conventional manner. The solid support thus bears at least two different capture agents, at least one for recognising each of the immunological markers to be detected. Preferably the capture agents are bound as a cocktail to a single area of the substrate, or to the whole substrate surface, within the well or vessel in which the test is to be conducted. Where the marker to be detected is an antigen, the capture agent should be an antibody against that antigen. Examples of antigens of interest, particularly in the context of screening blood

donations, include HBsAG, HIV P24Ag and HIV RTAg. Antibodies against these antigens are readily available.

Where the marker to be detected is an antibody, the capture agent may be an antigen recognised by that antibody. In this case there may be difficulty experienced in binding a mixture of the antigen with the antibody capture agents also to be used on the substrate. Thus it is generally preferred to use an antibody as capture agent for markers which are themselves antibodies. Suitable such capture agents include antibodies raised against class or sub-class specific epitopes on the marker antibodies and anti-idiotype antibodies raised against the antigen binding site of the marker antibodies. In general there should be no difficulty binding such antibody-capturing antibodies to the substrate as a mixture with antigen-capturing antibodies. Thus in a preferred aspect of the present invention all capture agents used are antibodies. Examples of antibodies of interest include anti-HBc and anti-HIV antibodies. Class- or sub-class-specific antibodies, and anti-idiotype antibodies against these antibodies, are readily available.

On incubation with the sample, for instance a body fluid such as whole blood, serum, plasma, saliva or urine, suspected to contain the immunological markers of interest, any immunological markers present in the sample bind to the respective immobilised capture agents and are thus immobilised on the support. Following optional washing to remove unbound material and the sample fluid, the solid support is probed using labelled binding partners for each of the immunological markers. Where the marker is an

antibody the binding partner may be the corresponding antigen or an anti-antibody antibody (especially an anti-idiotype antibody). Where an anti-antibody is used as binding partner, but the binding partner is not an anti-idiotype antibody, care must be taken to avoid detection of capture agents, non-specifically bound components of the sample and the like so as to avoid false positive results.

Where the marker is an antigen (for instance a microbial marker such as a viral or bacterial antigen) the binding partner will be an antibody against the antigen.

Direct detection of bound markers by labelled binding partners is preferred over competition-type detection to avoid possible false positive results.

Labelling of the binding partners, probing with the labelled binding partners and eventual detection of the label(s) are all achieved by conventional techniques. The labels (for instance radio-, enzyme or fluorescent labels) used on different binding partners may be the same, in which case all immunological markers will contribute to a single signal representing the sum of all the labels. Alternatively different labels may be used on the different binding partners facilitating the identification of individual immunological markers in the sample. The former version, where identical labels are used on all binding partners, is particularly advantageous where the test is to be used in routine screening of, for instance, blood donations. In this case the presence of any marker for a disease transmissible by blood transfusion (eg hepatitis viral antigens or antibodies, HIV antigens or antibodies) is

sufficient for the donation to be rejected.

The key to the invention is the use on the same solid support of a mixture of two or more capture agents (ideally antibodies) to capture the immunological markers. The mixture of antibodies may be obtained by a variety of techniques, for instance by recovery of immunoglobulins from hyperimmune serum from an animal immunised using a mixture of the immunological markers to be detected, by mixing individual monoclonal or polyclonal antibodies or by a combination of such techniques.

Those skilled in the art will readily be able to conduct tests in accordance with the present invention on the basis of their general knowledge and the above information.

One facet of the invention which requires attention is the balancing of the sensitivity of the various tests which are simultaneously conducted. Antibody assays are usually conducted in relatively highly diluted samples whereas antigen assays are conducted with neat samples or after only slight dilution. The format of the antibody capture and detection thus needs to be selected so as to be effective in relatively high concentration samples. Adjustment to accommodate this is within the ability of those skilled in the art.

The sample used in accordance with the present invention is preferably neat or is diluted only slightly, preferably not more than 1:10, more preferably not more than 1:5, for instance 1:4, 1:3, 1:2 or 1:1 dilution is applied. The degree of dilution may be selected within this range

having regard to the nature of the sample, the markers to be detected and the labelling and detection methods adopted. Increased dilutions may be selected to improve sensitivity especially where background reactions are reduced more rapidly by dilution than are the desired reactions necessary to complete the process of the invention.

Choice of diluent and the degree of dilution are matters within the competence of those skilled in the art. Often an appropriate detergent will be included in the diluent.

One advantage of the present invention is that it permits detection of both antibodies and antigens in the sample using only antibodies on the solid support. There is great practical difficulty in controlling and balancing mixtures of antibodies with antigens (especially in optimising the sensitivity of a test) which would otherwise have to be used to capture antibodies and antigens from the same sample.

Thus in a preferred aspect the present invention provides a process as defined above wherein the solid support bears a mixture comprising at least one antibody against an antibody or class of antibodies and at least one antibody against an antigen. The "antibody against an antibody or class of antibodies" may recognise the antigen binding site of an antibody (i.e. it may be an anti-idiotype antibody) or it may recognise a class specific determinant on antibodies and therefore recognise any antibody (irrespective of the nature of the antigen binding site of that antibody) from that class. For instance the antibody

against an antibody may be an anti-IgG antibody. It is possible to use polyclonal antibodies, even the immunoglobulin fraction from hyperimmune serum of an animal immunised with a combination of immunogens, as the capture agent, or one of the capture agents, in the invention since although there may be many irrelevant antibodies present, these will not interfere with the test in view of the eventual probing step. The ability to use a mixture of antibodies as capture agents on a single solid support permits optimisation of the test for maximum sensitivity simultaneously for a variety of different immunological markers.

The invention is illustrated by the following Examples:

Example 1

Screening for antibody against HIV (hereafter "anti-HIV") and for hepatitis B surface antigen (HBsAg).

i) Coat a solid phase with an antiserum which contains high titre antibodies against IgG and HBsAg.

ii) Incubate human serum with the solid phase allowing capture of any IgG and HBsAg on the solid phase.

iii) After washing, probe the solid phase with labelled (e.g. ^{125}I labelled) HIV antigen to detect anti-HIV within the captured human IgG and with identically labelled antibody against HBsAg (hereafter "anti-HBs") to detect captured HBsAg.

iv) Wash the solid phase and detect the presence of any label bound thereto.

A positive signal in step (iv) indicates the presence of one or both of HBsAg and anti-HIV.

Example 2

Screening for antibody against HBcAg (hereafter "anti-HBc")

i) Coat a solid phase (microtitre plate) with an antiserum which contains anti-Human IgG at 1μg/ml in a carbonate buffer (100μl/well).

ii) Incubate sample human serum (25 μl/well) for 30 minutes at 37°C with the solid phase and diluent 10mM citrate buffer pH 6.0 supplemented with detergent (200 μl/well) allowing capture of any human IgG on the solid phase.

iii) After washing, probe the solid phase with recombinant HBcAg conjugated to Horseradish peroxidase (diluted 1:100 using the same diluent plus 10% foetal calf serum (FCS) at 50 μl/well) to detect anti-HBc within the captured human IgG. Incubate for 30 minutes at 37°C.

iv) Wash the solid phase and develop using TMB liquid (commercially purchased tetramethyl benzidine substrate concentrate supplemented with citrate buffer and hydrogen peroxide (Murex Biotech Ltd)) (100 μl/well).

A significant increase in optical density in iv) indicates the presence of anti-HBc. The results are displayed in Table 1.

Table 1

Sample	O.D.
Anti-HBc positive	0.612
"	0.628
Normal human sera	0.111
"	0.033

Example 3

Screening for antibody against HBsAg (hereafter "anti-HBs").

i) Coat a solid phase (microtitre plate) with an antiserum which contains monoclonal anti-HBs at 0.26 μg/ml in a carbonate buffer (100 μl/well).

ii) Incubate sample human serum (25 μl/well) for 30 minutes at 37°C with the solid phase and diluent (as Example 2) (200 μl/well) allowing capture of any HBsAg on the solid phase.

iii) After washing, probe the solid phase by incubating with monoclonal anti-HBs conjugated to Horseradish peroxidase (diluted to 1:1500 in same diluent plus 10% FCS at 50 μl/well) for 30 minutes at 37°C.

iv) Wash the solid phase and develop using TMB liquid (as Example 2) (100 μl/well).

A significant increase in optical density in iv) indicates the presence of HBsAg. The results are displayed in Table 2.

Table 2

Sample	O.D.
HBsAg positive	0.935
"	0.855
Normal human sera	0.021
"	0.071

Example 4

Screening for antibody against hepatitis B (hereafter "anti-HBc") and for hepatitis B surface antigen (HBsAg).

i) Coat a solid phase (microtitre plate) with an antiserum which contains anti-human IgG (2 μg/ml) and monoclonal anti-HBs at 0.26 μg/ml.

ii) Incubate sample human serum (25 μl/well) with the solid phase and diluent (as Example 2) (200 μl/well) for 30 minutes at 37°C allowing capture of any human IgG and HBsAg on the solid phase.

iii) After washing, probe the solid phase with recombinant HBcAg conjugated to Horseradish peroxidase at 1:100 to detect anti-HBc within the captured human IgG and with identically labelled monoclonal anti-HBs at 1:1100 to detect captured HBsAg, diluted in same diluent plus 10% FCS. Incubate for 30 minutes at 37°C.

iv) Wash the solid phase and develop using TMB liquid (as Example 2) (100 μl/well).

A significant increase in optical density in iv) indicates the presence of one or both of HBsAg and anti-HBc. The results are displayed in table 3.

Table 3

Sample	O.D.
Anti-HBc only	0.876
"	1.077
HBsAg only	1.683
Anti-HBc and HBsAg	1.746
Normal human sera	0.006
"	0.062
"	0.050
"	0.077

CLAIMS

1. A process for testing for the presence of at least two immunological markers in a sample, at least one of which markers is an antigen, which process comprises incubating the sample with a solid support having immobilised thereon capture agents for each of the immunological markers so as to bind any of the immunological markers present in the sample to the solid support, contacting the solid support with labelled binding partners, for each of the immunological markers and detecting the presence of any label or labels bound to the solid support.

2. A process according to claim 1 wherein all the capture agents are antibodies.

3. A process according to claim 1 or claim 2 which further comprises a step of washing the solid support before contacting with the labelled binding partners.

4. A process according to any one of claims 1 to 3 which further comprises a step of washing the solid support before detection of the presence of any label or labels.

5. A process according to any preceding claim where a marker is an antibody and the binding partner is a corresponding antigen or an anti-antibody antibody.

6. A process according to any of claims 1 to 5 wherein a marker is an antigen and the binding partner is an antibody against the antigen.

7. A process according to any preceding claim wherein the label is chosen from a radiolabel, an enzyme

label or a fluorescent label.

8. A process according to any preceding claim wherein the solid support bears a mixture comprising at least one antibody against an antibody or class of antibodies and at least one antibody against an antigen.

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/GB 97/03289

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/53 G01N33/543

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP 0 781 997 A (FUJIREBIO KK ;AUBEX CORP (JP)) 2 July 1997 see claims 1,9-11 see column 8, line 9 - column 9, line 2	1-8
P,X	WO 97 01759 A (POPBB SNC ;TOLEDANO JACQUES (FR)) 16 January 1997 see claims see examples	1-8
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X	EP 0 484 787 A (BEHRINGWERKE AG) 13 May 1992 see claims 25-31 see page 12, line 1 - line 12	1-8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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11 March 1998	17/03/1998

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